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Intracellular pH during Calcification

A STUDY OF THE AVIAN SHELL GLAND

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The intracellular pH of the shell gland of the domestic fowl was calculated at various stages in egg-shell formation. The calculation is based on the distribution of 5,5-dimethyloxazolidine-2,4-dione between intracellular and extracellular water. The results show a rapid fall in intracellular pH at the time of mineralization and this is interpreted as indicating a removal of protons from the site of calcification.

The mineralized deposits of vertebrates consist of the calcium salts of the principal inorganic buffers of the body, i.e. the carbonate and phosphate systems. The properties of these ions that make them particularly suitable for forming both buffering systems and crystalline structures have been discussed by Wilbur & Simkiss (1968). However, since the anions involved exist in most of the body fluids in the monohydrogen form the process of calcification must, in some way, involve the removal of protons. It is most likely that this process occurs near the site of crystallization, although other possibilities can easily be envisaged. It was therefore decided to attempt to study intracellular changes in acid–base balance at sites of calcification.

One of the most fruitful suggestions about active changes in cellular pH is that it can be regarded as involving the separation of water into protons and hydroxyl ions (Davies, 1948). The possible biochemical reactions involved in this process have been discussed by Bittar (1964) and its importance has been commented on by Caldwell (1956). Thus the oxyntic cells of the gastric mucosa, which secrete acid, become alkaline in the process, whereas active pancreatic cells, which form an alkaline secretion, become acid. It would be expected therefore that if calcification involves the formation of an alkaline secretion the cells responsible should show a fall in intracellular pH.

Investigations into the process of mineralization have been hampered by two difficulties. First, it usually occurs in a 'micro-environment' around individual cells that therefore makes many analyses impossible, and, secondly, it is difficult to obtain pure samples of the cells since they are often dispersed and embedded in mineral deposits as well as being in various states of activity. It was considered that many of these difficulties might be avoided by studying the mineralization of the avian egg shell rather than, for example, the ossification of cartilage.

The avian shell gland is a relatively simple cellular structure consisting of a ciliated epithelium overlying a mass of tubular glands (Richardson, 1935). In the domestic fowl it secretes up to 6g. of calcium carbonate within a period of 20hr. The calcification of the egg shell is easily followed by palpating the bird and it was therefore decided to investigate the intracellular pH of the shell gland at various stages during egg-shell calcification.

MATERIAL AND METHODS

Two breeds of domestic fowl were used in this work, namely Thornber's 404 and Arbor Acres. Most of the work was performed on the Thornber's strain but there was no apparent difference between the results obtained from the two strains (Tables 1 and 2). All the birds were laying regularly at the time of the experiments; they were housed in normal cages and fed on a commercial laying ration (Paul's no. 2).

Intracellular pH was measured in the shell gland at various stages of the laying cycle of 22 birds. The stage of shell formation was roughly calculated from the timing of previous ovipositions, and the birds were then palpated via the cloaca and rectum so that a more precise time could be obtained for the entry of the egg into the shell gland. Birds were selected at various stages in the laying cycle and 5ml. of blood was removed from the brachial vein of each into a heparinized syringe. The pH of the freshly drawn blood was measured in a Radiometer micro-electrode system at 38°, as was the pH of similar blood samples equilibrated in an Astrup microtonometer with analysed samples of CO_2+O_2 (1:25 and 2:25). The pH of blood is linearly related to the log pCO_2 so that it is possible to calculate the pH and pCO_2 values of the blood *in vivo* from these readings by using the methods of Sigaard-Andersen (1965). The remaining sample of blood was centrifuged to produce what is referred to below as 'control plasma'.

Immediately after the withdrawal of the 5ml. sample,

5ml. of 0·9% NaCl containing 600mg. of inulin, 20mg. of thiocyanate and 200mg. of DMO* was injected into the brachial vein. After a period of 1hr. the bird was killed and samples of plasma, pectoralis muscle and shell gland were collected. The stage of reproduction was noted, and in cases where the shell calcification was well advanced the egg and shell were weighed. The tissues removed from the bird were weighed and immediately homogenized with a known volume of water in a Silverson blender.

Intracellular pH was calculated from the equation:

$$pH_i = pK' + \log\left\{\left[\frac{C_t}{C_e}\left(1+\frac{V_e}{V_i}\right)-\frac{V_e}{V_i}\right]\times[10]^{pH_e-pK'}+1\right]-1\right\}$$

where pH_i is the intracellular pH, pH_e is the extracellular pH, C_t is the concentration of DMO in total tissue, C_e is the concentration of DMO in extracellular fluid, V_e is the volume of extracellular water, V_i is the volume of intracellular water and pK' is the apparent ionization constant of DMO (6·13).

The deduction of the equation and its application are given by Waddell & Butler (1959). The DMO was extracted from the tissues by using 5M-NaH_2PO_4 and ether followed by borate buffers. It was measured by recording the extinctions in buffer, pH 9·0, and in buffer plus 0·1N-HCl, with a Hilger and Watts u.v. spectrophotometer at two wavelengths, 215 and 220mμ. The extinction at pH 9·0 was corrected by subtracting the extinction in acid solution, and the concentration of DMO was derived from the difference between the corrected extinctions at 215 and 220mμ. Fresh standards were used in every experiment and treated identically throughout the extraction procedure. Known amounts of DMO, inulin, chloride and thiocyanate were added to some of the muscle and shell-gland samples to check their recovery and to ensure that there was no binding of these materials to tissue components.

The total water content of the tissues was determined by drying weighed samples for at least 12hr. in an oven at 120°. The distribution of this water between intracellular (V_i) and extracellular (V_e) partitions was determined by three independent methods based on tissue and plasma concentrations of endogenous chloride or exogenous thiocyanate or inulin. Chloride was determined in nitric acid digests of the tissue by titration with 0·01N-$AgNO_3$ in 1·5N-H_2SO_4 in a Radiometer autotitrator with an Ag/Hg_2SO_4 reference-electrode system by a variation of the method of Cotlove, Trantham & Bowman (1958). Thiocyanate was determined colorimetrically as ferric thiocyanate by using a Spectrochem spectrophotometer at 470mμ and with standards made up in 'control plasma'. Inulin was hydrolysed and determined colorimetrically with resorcinol at 480mμ in a Spectrochem spectrophotometer (Varley, 1963).

In some experiments either the thiocyanate or the inulin was omitted from the injection solution. There were also some experiments in which 2hr. was allowed between the time of injection and killing to check that equilibrium of the reagents in the body was complete. All experimental analyses were done in duplicate.

*Abbreviation: DMO, 5,5-dimethyloxazolidine-2,4-dione.

RESULTS

The mean pH of the plasma of the birds used in these experiments was 7·285 ± 0·529 at a mean pCO_2 of 43·71 ± 2·12mm. Hg.

The complete data for the calculation of intracellular pH in each bird are rather extensive and a full analysis of the measured and calculated values is therefore only given for five birds (Table 1). The shell gland contains 84% of water as compared with the muscle, which contains 73%. About 56% of this water is extracellular in the shell gland, as compared with about 17% in muscle. The exact distribution between intracellular and extracellular fractions varies slightly with the method of measuring these spaces. There is, however, no consistent trend for any one indicator to give larger values than another in the shell-gland data. It was therefore decided to derive the intracellular pH in each case and take the mean of the two closest calculated values to obtain a representative figure of intracellular pH. The mean of the variation between all these values was 0·095 pH unit and for most of the calculations it was about 0·050 pH unit. These results are shown in Table 2 and Fig. 1.

The mean value of the intracellular pH of the shell gland during the period from 2 to 10hr. of calcification was 6·531 ± 0·387 whereas the value for the last 8hr. (i.e. from 10 to 18hr.) was 6·970 ± 0·223. The difference was significant ($P < 0·01$) as assessed by Student's t test. Corresponding values for the intracellular pH of pectoralis muscle were 6·795 ± 0·026 and 6·877 ± 0·125. The difference between these values was not significant.

The equation for a straight line through the values for intracellular pH of the shell gland during the time of shell formation is $y = 6·387 + 0·0379x$ (Fig. 1). The correlation coefficient is highly significant ($P < 0·001$).

A number of variations in the experimental technique were undertaken. There was no apparent difference in the calculated intracellular pH of the shell gland or muscle when the injections were given 2hr. to equilibrate within the bird instead of the usual 1hr. (bird T02, Table 1; bird T04, Table 2). Similarly, omission of the thiocyanate from the injection solution had no obvious effects on the results (birds A28 and T04, Table 2).

Mean values for the recovery of DMO added to tissue homogenates were 97·0% for muscle and 101·9% for shell gland. Similar values for the recovery from shell-gland homogenates of added chloride were 102%, for thiocyanate 95% and for inulin 101%.

DISCUSSION

The technique of measuring intracellular pH by means of the drug DMO has been widely used since

Table 1. *Measured and calculated values involved in determining intracellular* pH *in shell gland and muscle*

The values are selected from various experiments to indicate the influence of (1) different stages of shell formation, (2) the variations found in using three different indices for measuring intracellular and extracellular fluid spaces and (3) the effect of varying the time available for equilibrium of injected substances within the bird. Bird numbers beginning with A are Arbor Acres; those commencing with T are Thornber's 404.

			Measured values								Calculated values								
											V_e			V_i			pH_i		
Tissue	Bird no.	Calcification time (hr.)	DMO (μg./g.)	Cl^- (m-equiv./kg.)	SCN^- (μg./g.)	Inulin (μg./g.)	Water (g./g.)	pH_e†	C_t (μg./g.)	C_e (μg./g.)	Cl^-	SCN^-	Inulin	Cl^-	SCN^-	Inulin	Cl^-	SCN^-	Inulin
Shell gland	A26	2	154·7	59·4	10·2	624·0	0·84	—	184·1	268·3	0·48	0·52	0·53	0·36	0·32	0·31	6·66	6·40	6·31
Plasma	A26	2	235·1	109·0	17·0	1023·4	0·92	7·30											
Shell gland	T16	5½	126·7	47·8	13·4	370·2	0·82	—	150·82	233·1	0·37	0·44	0·45	0·47	0·40	0·39	6·75	6·57	6·52
Plasma	T16	5½	202·0	108·5	26·3	714·6	0·91	7·24											
Shell gland	A30	12	152·2	54·7	9·6	282·2	0·84	—	181·2	228·0	0·43	0·45	0·41	0·41	0·39	0·43	7·06	7·05	7·08
Plasma	A30	12	197·6	110·9	18·6	603·4	0·91	7·33											
Shell gland	T02*	12	155·4	66·2	13·1	184·6	0·84	—	185·0	222·8	0·53	0·46	—	0·31	0·38	—	7·03	7·09	—
Plasma	T02*	12	195·2	109·1	24·7	246·4	0·92	7·32											
Shell gland	T09	16	166·5	75·7	10·2	220·0	0·83	—	200·6	227·0	0·57	0·47	0·44	0·26	0·36	0·39	7·07	7·15	7·16
Plasma	T09	16	198·9	115·8	19·0	433·2	0·92	7·30											
Muscle	A26	2	99·4	8·8	2·9	188·8	0·72	—	138·1	268·4	0·07	0·15	0·16	0·65	0·57	0·56	6·93	6·83	6·82
Muscle	T16	5½	85·4	16·9	2·1	716·4	0·73	—	117·0	233·1	0·13	0·07	—	0·60	0·66	—	6·77	6·85	—
Muscle	A30	12	92·7	12·4	2·8	345·8	0·73	—	127·0	228·0	0·10	0·13	—	0·63	0·60	—	6·99	6·96	—
Muscle	T02*	12	88·6	21·1	2·7	196·4	0·74	—	119·8	222·8	0·17	0·10	—	0·57	0·64	—	6·88	6·96	—
Muscle	T09	16	92·7	21·8	2·7	215·2	0·73	—	127·0	227·0	0·17	0·12	—	0·56	0·61	—	6·89	6·94	—

* Equilibration for 2 hr. between injecting and killing birds.

† pH_e assumed to be 0·02 unit higher than plasma pH (Waddell & Butler, 1959).

Table 2. *Mean values of intracellular* pH *of shell gland and pectoralis muscle from birds at various stages of shell mineralization*

Bird numbers beginning with A are Arbor Acres; those beginning with T are Thornber's 404

Bird no.	Time in shell gland (hr.)	Intracellular pH (shell gland)	Mean ± S.D.	Intracellular pH (pectoralis muscle)	Mean ± S.D.
T19	(ovulation)	7·02		6·94	
A53	(magnum)	7·01		—	
A27	(isthmus)	6·66		6·93	
T56	2	6·72		—	
T59	2	6·76		—	
A26	2	6·36		6·82	
T08	2	6·52		6·95	
T04*	3	6·22		6·75	
T11	4	—	6·53 ± 0·39†	6·74	6·80 ± 0·03‡
T21	5	6·35		6·74	
T16	5½	6·55		6·81	
A54	8	6·37		—	
A28*	8	6·75		6·86	
T10	10	6·52		6·69	
A24	11½	7·05		6·86	
A30	12	7·07		6·98	
T02	13	7·06		6·92	
A31	14	7·06		6·98	
T17	14	6·56	6·97 ± 0·22†	6·73	6·88 ± 0·12‡
T15	15	6·74		6·72	
T20	15	7·05		6·92	
T09	16	7·15		6·91	

* No thiocyanate used in injection solution.
† Difference significant at $P < 0.01$.
‡ Difference not significant.

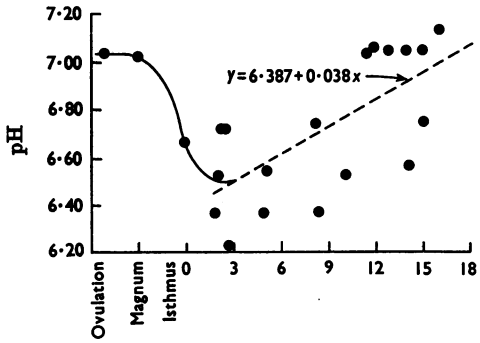

Fig. 1. Intracellular pH of the shell gland at various stages in the reproduction cycle. The values obtained during the time the egg is actually in the shell gland itself have been used to calculate the equation of a straight line. This is represented by the broken line.

its introduction by Waddell & Butler (1959) (see Butler, Waddell & Poole, 1966). The method involves a number of assumptions that have been considered in detail elsewhere (Roos, 1965; Clancy & Brown, 1966). The most important of these are the time taken for equilibration of the injected materials, the way in which they are distributed in the body and the meaning of the value calculated as intracellular pH.

Equilibration of the drug within the body appears to be rapid and is effectively complete within the 1 hr. allowed by most workers. An equilibration time of 2 hr. did not have any influence on the calculated pH of the shell gland. It was not favoured, however, because it made the timing and interpretation of the experiment more difficult, especially since the intracellular pH of the shell gland itself appears to vary with time. This variation in intracellular pH is presumably due to some particular cells in the tissue and it is not suggested that the values recorded are the actual pH of the tissue. It is in fact likely that even different regions of the same cell have different pH values. Thus the values recorded in these experiments have no absolute significance and are mainly of interest because they show that the average pH of the whole tissue becomes more acid at the start of calcification and then progressively less so as mineralization continues.

The calculation of intracellular pH is very

dependent on the determination of intracellular and extracellular water. The size of these compartments varies slightly with the method used to determine, or define, them. Thus the chloride ion has been widely used in studies on muscle since it is mainly extracellular in this tissue. The distribution of this ion in the shell gland is not known, but the results of the present work indicate that it is also largely extracellular in this tissue. The situation is complicated, however, since the shell gland also secretes a so-called 'plumping fluid' during the time of egg-shell formation. This fluid contains chloride ions (Jack & Lake, 1967) so that the concentration within the gland may vary during the time of shell secretion. Thiocyanate was found to be a very good indicator of extracellular fluid, although it has the disadvantage that it has been shown to inhibit acid secretion in the stomach (Feldberg, Keilin & Mann, 1940). The concentrations used in the shell-gland experiments were, however, well below those that normally produce any side effects and omission of this ion from the injection solution did not appreciably affect the intracellular pH (Table 2). As a further check of extracellular fluid volumes inulin was also used as a marker. This material is, of course, excreted by the kidneys, so that it is again important to keep the time available for equilibration as short as possible. All three of these indicators of extracellular spaces could therefore produce some variations in interpretation, but this is obviously inevitable and rests to some extent on the definition of extracellular space. It was therefore decided to compromise and take as a representative figure the means of the two closest values of intracellular pH based on the different measures of extracellular water. The variation involved in the different methods is, however, normally small and similar results to those shown in Fig. 1 can be obtained by taking the data from individual indicator substances.

The calcification of the egg shell begins at about the time that the egg passes out of the isthmus and into the shell-gland region of the oviduct (Simkiss, 1968). The rate of mineralization is normally slow for the first 1–2hr. but proceeds at a uniform rate for about 16hr. and then slows down or stops in the last 1–2hr. before oviposition. It is difficult to determine the exact timing of the start of the shell formation and to discuss the changes that might occur it was arbitrarily decided to divide the time of calcification into two equal periods of 2–10 and 10–18hr. There is a large and significant difference in the intracellular pH of the shell gland in these two periods (Table 2). It is apparent that as shell formation starts the intracellular pH falls from a value of about 7·0 to about 6·5. The changes that follow this are probably complicated by two phenomena, namely, the continual formation of hydrogen ions and their passage out of the shell gland and into the bloodstream. The changes in intracellular pH with time can, however, be fitted to a straight line with a statistically significant correlation. This has been done in Fig. 1, which indicates the tendency for the intracellular pH to rise towards resting values as shell formation continues.

The mechanism by which the shell gland forms the carbonate ion of the eggshell is unknown. It was suggested by Gutowska & Mitchell (1945) that two bicarbonate ions were used to form a carbonate ion and a molecule of carbonic acid, which was then broken down by carbonic anhydrase to form carbon dioxide and water. An alternative suggestion is that the carbonate ion is formed directly from carbon dioxide, to which the cells are presumably more permeable (Simkiss, 1961; Hodges & Lorcher, 1967). Neither theory has any decisive experimental backing, although it is known that during shell formation there is a fall in the pH and bicarbonate concentration of the blood followed by hyperventilation of the bird and a lowering of its carbon dioxide partial pressure (Mongin, 1968). The present work indicates that there is a rapid fall in intracellular pH when shell formation starts. It is suggested that this may represent the separation of water into hydroxyl ions, which are secreted into the lumen of the gland, and protons, which cause the fall in intracellular pH. The protons will eventually pass into the blood, causing the decline in pH and bicarbonate concentration that have already been mentioned. The hydroxyl ions could react with carbon dioxide, under the influence of carbonic anhydrase, which exists in the shell gland (Common, 1941), to form first bicarbonate and then carbonate ions in the lumen of the shell gland. The process is most vigorous at the onset of shell formation, and this would be in keeping with the suggestion that larger ionic products are necessary for the nucleation of the crystals of the shell as opposed to their later growth (Simkiss, 1968). The later continual rise in pH is perhaps somewhat surprising, and it should be realized that, although the points can be fitted to a straight line, other interpretations are possible and there may be a much sharper rise in pH towards the end of calcification. Further work will obviously be necessary to test these hypotheses, but it appears likely from the present work that the net effect is a removal of protons from the site of calcification. Whether this is a general characteristic of all forms of mineralization, as suggested in the introduction, is again a possibility that can only be answered by further work.

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